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Myosin light chain and calcium regulating protein differences in chronic musculoskeletal neck and shoulder pain

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ABSTRACT

Proteomic screening analysis has detected myosin light chain (MLC) as a protein implied to be involved in chronic musculoskeletal neck and shoulder pain. Several analyses of MLC proteins have stated a difference in phosphorylation being the determining factor for protein activation hence altered contractility of the muscle in i.e. senescence. In continuation of a previous publication, this study is an attempt to analyze the different MLC isoforms by mass spectrometry and immune-analyses in myalgic and healthy trapezius muscle. In the present study no differences in phosphorylation level between the corresponding individual proteins were detected using LC-MSMS and immunoblotting; instead we assigned different isoforms of regulatory MLCs. To further elucidate the contractility: calcium (Ca²⁺) regulatory proteins, sarco (endo)plasmic reticulum Ca²⁺ ATPase 1 (SERCA-1) and calsequestrin (CSQ) were analyzed by western blot. The analysis revealed a significantly increased abundance of SERCA-1 protein in the myalgic muscle and a significantly increased abundance of CSQ in healthy muscle. Myalgic muscle contraction patterns have in previous studies shown to differ from healthy muscle which may be connected to the Ca²⁺ availability in the muscle. Here we present the proteomic characterization of differences in Ca²⁺ regulating proteins and particularly regulatory MLCs in trapezius muscle of women with chronic musculoskeletal neck and shoulder pain.

Keywords: calcium (Ca²⁺), mass spectrometry, phosphorylation, muscle pain, myosin light chain, trapezius.

Abbreviations

MLC: myosin light chain; **MHC:** myosin heavy chain; **SERCA 1:** sarco(endo)plasmic reticulum Ca²⁺ ATPase 1; **CSQ:** calsequestrin; **two dimensional – differential gel analysis:** 2D-DIGE; **liquid chromatography mass spectrometry mass spectrometry:** LCMSMS.

1. Introduction

The introduction of proteomics and metabolomics to the field of work related musculoskeletal disorders [1, 2] have suggested a difference in a number of proteins and metabolites relating to chronic musculoskeletal neck and shoulder pain (or chronic trapezius myalgia). These screening studies have presented proteins correlating to an altered metabolism, different contractibility and inflammatory and acute stress responses to differ in abundance between myalgic and healthy muscle [3].

Regulatory myosin light chain 2 (MLC2) was one protein appearing more abundant in chronic trapezius myalgia, according to a proteomic systematic explorative screening of the present biopsy material [2]. MLC2 has been a protein of interest in previous studies concerning sarcopenia (ageing muscle) [4-6] and muscle inactivity, such as during bed rest [4, 7, 8]. Phosphorylation of MLC2 has previously shown to affect the calcium sensitivity of skeletal muscle contraction [9], affecting the muscles ability to contract [10]. Fast regulatory MLC2 traditionally correlate with fast contracting type 2 fibers in skeletal muscle [11]. During low load work it

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has been shown that the muscle contraction in myalgic muscle is activated by a higher contribution of type 2 motor units compared to healthy muscle [12]. These several electromyographic studies indicate altered activation patterns of the trapezius muscle in subjects with chronic neck-shoulder pain conditions [13-15]. There is also discussed whether painful muscles are disused and clinically deconditioned [16]. One or both of these circumstances may be associated with altered contractility. A better understanding of molecular events related to contractility aspects can facilitate the development of targeted treatment interventions in subjects with chronic myalgia. Introducing proteins of Ca²⁺ regulation into this area of research may explain the excitation-coupling properties of the myalgic muscle. The result from the proteomic study suggesting the Ca²⁺ regulatory MLC2 fast to be more abundant in myalgic muscle [2], are one protein that may explain the differences in muscle contractility. The muscular symptoms associated with chronic trapezius myalgia are stiffness, tension and a decreased maximal force torque rate compared to healthy muscle [17, 18]; and also the inability to relax the muscle during the naturally occurring pauses during dynamic work [19, 20].

In muscle contraction Ca²⁺ is regulating the contractility of the muscle and the regulatory myosin light chains, situated on the myosin head regulates the calcium sensitivity of the myosin molecule [21]. Sarco(endo)plasmic reticulum Ca²⁺ATPase 1 (SERCA-1) transfers Ca²⁺ from the cytosol of the cell to the lumen of the sarcoplasmic reticulum. SERCA-1 is predominately abundant in type 2 fibers [22]. Calsequestrine (CSQ) is the most abundant Ca²⁺ binding protein of the sarcoplasmic reticulum in skeletal muscle [23]. CSQ releases Ca²⁺ upon muscle contraction and are available in two different isoforms, CSQ1 predominantly in type 2 muscle fibers and CSQ2 predominantly in type 1 [23]. CSQ buffer Ca²⁺ in the sarcoplasmic reticulum thus assisting SERCA to not having to pump Ca²⁺ against a high concentration gradient [24]. Accumulation of Ca²⁺ in the muscle has been shown in rats exposed to prolonged chronic low frequency muscle stimulation and is hence proposed to be a mechanism behind chronic muscle pain [25, 26]. The exact mechanisms behind this accumulation is yet not known, but serves as one of many hypothesis [27] behind the underlying pathophysiological mechanisms of chronic trapezius myalgia.

This study aims to characterize on proteomic level changes in Ca²⁺ regulating proteins in trapezius muscle of women with chronic trapezius myalgia and, in particular, to verify the possible difference in abundance of the phosphorylation of regulatory myosin light chain protein in healthy and myalgic trapezius muscle.

2. Material and Methods

The subjects, clinical examination, biopsy collection and

Two-Dimensional Difference Gel Electrophoresis (2-D DIGE) image analysis are presented in a previously published paper presenting a proteomic screening analysis of myalgic and healthy muscle [2]. In short the 2D-DIGE method was applied on frozen muscle samples that were suspended in lysis buffer and homogenized followed by protein quantification. Labelling of protein samples was made with CyDye minimal dyes, Cy2, Cy3 and Cy5, in accordance to manufacturers' protocol. The internal standard method was used [28]. The samples were applied onto 24 cm 3-11 Non-Linear (NL) Immobilised pH gradient (IPG) strips and rehydrated at room temperature in the dark for 16 hours. The first dimension was run using Amersham Ettan™ IPGphor unit. Prior to the second dimension the gel strips was equilibrated and alkylated. The second dimension was conducted by using the Ettan DALT six apparatus. Gels were then immediately scanned with a Typhoon™ 9410 scanner. The scanned image was further processed using ImageQuant™ V5.2 and DeCyder™ V6.5. The 2D-DIGE protocol used has previously been evaluated [29]. A more detailed description of the analysis is available in Hadrévi et al 2013 [2].

Additional analysis and identification of the myosin light chain protein spots appearing on 2D-gels that according to a number of publications previously presented [4, 6, 7] are to be separated due to their individual level of phosphorylation. The main objective of our study is to identify the differences in phosphorylation between the different protein spots separated on a 2D-gel that are of interest in the previous screening analysis [2], by utilizing phosphorylation analysis and high resolution mass spectroscopy, in accordance to the methods presented below:

2.1. Phosphorylation analysis

A preparative gel (600 µg protein) stained with Sypro Ruby was used for analysis by mass spectrometry. 10 protein spots corresponding to the different isoforms of myosin light chains were excised and in-gel digestion was performed according to previously described protocols [30]. The samples were analyzed by mass spectrometry. 200 µg proteins were separated by 2D-PAGE. The ten protein spots corresponding to the different isoforms of myosin light chains were transferred from the gel by electroblotting to a PVDF membrane, blocked and incubated overnight with phosphor-threonine or phospho-tyrosine antibody (Cell Signaling Technology, Danvers, MA). Immunoreactive protein spots were detected using ECL prime detection system (GE Healthcare).

2.2. High-Resolution Mass Spectrometry

The obtained peptide mixtures were analyzed by LC-MS/MS. Separation was done using nano-flow HPLC system (EASY-nLC II, Thermo Fisher Scientific) and data were

acquired using LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific). Peptides were separated during 90 min by reverse phase chromatography on a 20 mm×100 µm C18 pre column followed by a 100 mm×75 µm C18 column (particle size 5 µm, NanoSeparations, Netherlands) at a flow rate 300 nL/min. A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was distributed as follows: starting with 3% B; linear gradient 3%–35% B in 0–60 min; 35%–90% B in 60–90 min. Automated online analyses were performed with LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific) with nano electrospray source; 240°C capillary temperature; spray voltage, 2200 V. MS spectra were acquired in profile mode by FTMS at resolution of 30,000 (at m/z 400). Top 20 most intense multiply charged ions from were selected with an isolation window of 2.0 and fragmented in the linear ion-trap by collision induced dissociation with normalized collision energy of 30. Dynamic exclusion of sequenced peptides for 60 s and charge state filtering disqualifying singly charged peptides were activated and predictive AGC was enabled. Centroid mode was used for CID MS/MS.

2.3. Database Searches and Data Interpretation

Obtained data files were analyzed by Proteome Discoverer 1.4 (Thermo Fisher Scientific) and the search algorithm SEQUEST was used against UniProt release 2013_09 Homo sapiens database. Carbamidomethylation of cysteine residues was set as static modification, and phosphorylation of threonine and serine and oxidation of methionine were set as variable modifications. Trypsin was used as the enzyme with 2 missed cleavages allowed. The precursor mass tolerance was set to 10 ppm with 0.6 Da fragment mass tolerance. Proteins were identified with at list 2 peptides of rank 1 with high score and target false discovery rate 0.01.

2.4. SERCA-1 and Calsequestrin analysis

Muscle biopsies were heat stabilized using Stabilizer T1 device (Denator AB, Sweden). The biopsies were homogenized in lysis buffer (Protein Simple, Santa Clara, CA) and by sonication 3 × 10 s. The samples were stored for 2 h at 4°C and then centrifuged for 1 h at 4 × 10⁴ g at 5°C. The supernatant was then removed to a new tube. Total protein concentrations were measured by 2-D Quant kit (GE Healthcare) according to the manufactures recommendation. Samples were diluted to adjust protein concentration to 1 µg/µl in 5 µl with sample buffer. The quantification of expression level of SERCA-1 was performed by a novel nanovolume capillary electrophoresis based protein analysis, Simple western size/charge assay instrument Peggy™ [31]. The lysate samples were mixed with a master mix containing 80 mM DTT, fluorescent molecular markers and heated at 95°C for 10 minutes. The samples, blocking reagents, primary antibody, secondary antibody

and chemiluminescence substrate were loaded onto a 384-well plate according to the user manual provided by the company (Protein Simple, Santa Clara, CA). The primary antibody (SERCA-1; Abcam, Calsequestrin; Novus Biological) was diluted in antibody diluent in a 1:50 dilution. The chemiluminescence signals were digitized using a charge coupled camera (CCD). The digital images were analyzed with Compass software (ProteinSimple), and the quantified data of the detected protein were reported as molecular weight and peak area.

2.5. Statistical analysis

The proteomic data was analyzed using a multivariate projection model, projections to latent structures with discriminant analysis (PLS-DA) [32]. PLS-DA is a supervised linear regression method used to find a relationship between X variables (spot volume ratios) and a binary Y vector by using class membership data (MYA/CON). The spot volume ratios were mean centered and scaled for unified variance. The number of PLS-DA components was determined by full cross-validation (SIMCA-P version 12, Umetrics AB, Umeå, Sweden). The PLS-DA model was also validated by both response permutation tests and CV-ANOVA on the residuals [33]. To identify spot of interest variable of importance value (VIP)>1 was used.

For the traditional statistical analyses IBM SPSS (version 22) was used. Mann-Whitney U test was used for the group comparisons of SERCA-1 and CSQ. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Myosin light chains

Proteins of interest in this analysis are MLC proteins and adjacent protein spots in the 2D gel analysis presented in figure 1A. Previous studies have presented the 2D-gel protein pattern of human skeletal muscle [34] and the possible correlation between a regulatory MLC and myalgia [2]. PLS-DA of the 2D- protein pattern from 12 female cleaners with work-related trapezius myalgia and 12 pain free female cleaners reveals the importance of the ten investigated proteins in separating between MYA and CON. Proteins with VIP>1 were, two fast regulatory MLC, *H3BN54* and *H3BML9* (VIP 1.41 and 1.04 respectively) and one slow MLC *Q6IB42* (VIP 1.09) (figure 1B). The other proteins showed VIP<1. The ten protein spots (figure 1A) were analyzed by mass spectrometry. The results showed that the ten spots were different proteins with different molecular weight and pI. The sequences of each individual protein isoform, obtained from the LC-MS analysis are here presented as supplement information. Phosphorylated peptides were detected at one position in spot 4-5 (table 1). There were no phosphorylated peptides in spot 6-10. The

Table 1. Myosin light chain (MLC) protein isoforms identified from trapezius muscle by 2D gel and following mass spectrometry analyses. The spot numbers are referred to the marked spots in figure 2. Sequenced phosphorylated peptides are shown. VIP = variable of importance in projection from the PLS-DA analysis (figure 1B).

| Spot no. | Identified protein | Uniprot. Acc. no. | pI # | Mw # (kDa) | Seq.Cov. (%) | Phosphorylated peptides | VIP |
|----------|---|-------------------|------|------------|--------------|-------------------------|------|
| 1 | Fast skeletal MLC2 regulatory | H3BML9 | 5.21 | 15.6 | 72.9 | TVEGGsssVFsMFDQTQIQEFK | 1.04 |
| 2 | Fast skeletal MLC2 regulatory | H3BN54 | 5.01 | 15.6 | 73.3 | TVEGGsssVFsMFDQTQIQEFK | 1.41 |
| 3 | Fast skeletal MLC2 regulatory | Q96A32 | 4.90 | 15.6 | 77.5 | TVEGGsssVFsMFDQTQIQEFK | 0.56 |
| 4 | Slow MLC, ventricular/ cardiac isoform | G3V1V8 | 5.31 | 18.0 | 67.1 | AGGANsNVFsMFEQTQIQEFK | 0.65 |
| 5 | Slow MLC, ventricular/cardiac isoform | P10916 | 5.21 | 18.0 | 67.5 | AGGANsNVFSMFEQTQIQEFK | 0.26 |
| 6 | Slow MLC, ventricular/ cardiac isoform | Q61B42 | 5.10 | 18.0 | 48.6 | | 1.09 |
| 7 | Ventricular/ slow twitch MLC alkali | P08590 | 5.41 | 22.1 | 57.4 | | 0.88 |
| 8 | MLC 1/3, skeletal muscle isoform | P05976 | 5.35 | 22.1 | 76.8 | | 0.41 |
| 9 | MLC3 of MLC 1/3, skeletal muscle isoform | P05976-2 | 5.32 | 22.1 | 80.0 | | 0.45 |
| 10 | Tropomyosin beta chain | P07951 | 5.30 | 22.1 | 74.9 | | 0.99 |

Calculated.

In the present study, myalgic muscle exhibits a significantly increased abundance of SERCA-1 compared to healthy muscle (figure 2A). The SERCA-1 protein has shown to exist in both types of fibers but in a considerably higher abundance in type 2 fibers [51]. The increased abundance of SERCA-1 in myalgic muscle indicates a difference in the contractile characteristics of myalgic muscle compared to healthy muscle. Previous studies have shown an impaired ability to relax the myalgic muscle during the pauses that are occurring in a standardized dynamic work [20]. Since the SERCA-1 proteins are determinants of the Ca^{2+} influx and *efflux* of the muscle sarcolemma, where the efflux is crucial for the muscle relaxation rate. In this material where there is no difference in fiber type content [52], though the high amount of SERCA-1 is indicative of fast twitch characteristics of myalgic muscle as it resequesters Ca^{2+} enabling a fast relaxation of the muscle. When CSQ subsequently binds Ca^{2+} it will buffer and keep the Ca^{2+} comparatively low and still have a large amount of Ca^{2+} ready to be released to produce muscle contraction [23, 41]. SERCA also enables leakage out of the SR and since the Ca^{2+} uptake by SERCA is energy consuming where a higher concentration of ADP will reduce Ca^{2+} uptake in the SR [24].

Fast twitch fiber normally generates force in a rapid manner, in comparison to slow twitch type 1 fibers. Fast twitch fibers hence needs to have a substantial amount of Ca^{2+} stored in the SR to be released rapidly upon stimulation [53] in order to saturate the calcium binding sites on troponin C. Recent laboratory investigations have shown a reduced rate of torque development in patients suffering from trapezius myalgia [17, 18]. CSQ is a protein that buffers Ca^{2+} in order to upon strong stimuli release Ca^{2+} to enable muscle contraction. Type 2 fibers have been shown to be more dependent on CSQ availability compared to type 1, due to their specific contractile properties [54]. CSQ knock-out mice show a reduced ability to quickly generate maximal force during muscle contraction [54-56], similar the muscular traits of chronic muscle myalgia [17, 18, 20]. Also, CSQ could be connected to an increased fatigability of the myalgic muscle. The lower abundance of CSQ in myalgic muscle compared to healthy muscle may explain the muscular traits of the myalgic muscle although this specific correlation has not yet been presented.

Also, a proposed pain inducing calcium accumulation could be obtained by a lack of buffering capacity in the SR and energy deficits disturbing ATP consuming SERCA

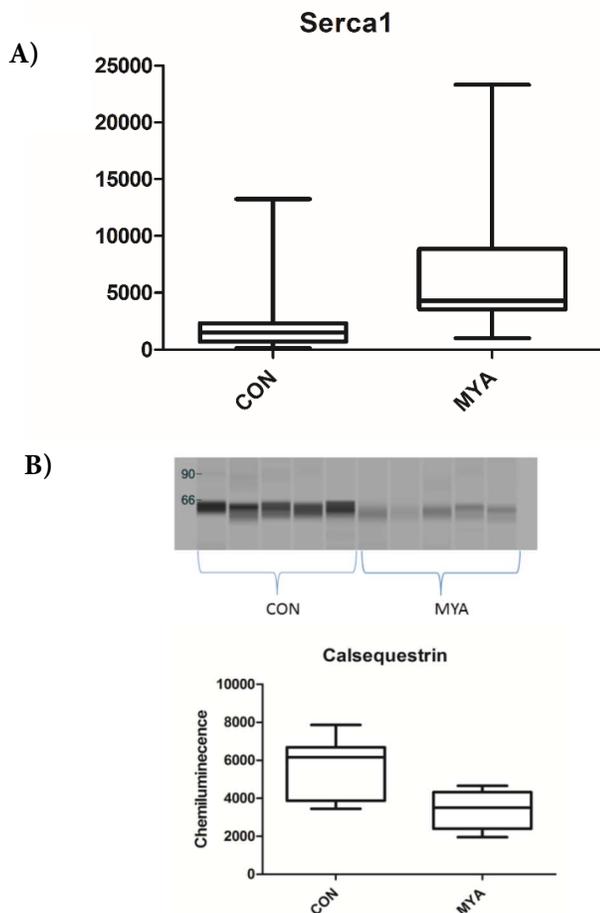


Figure 2: A) SERCA-1 significantly more abundant in myalgic muscle (MYA n=11) compared to healthy (CON, n=11) $p=0.017$. B) CSQ is significantly less abundant in myalgic muscle (MYA) compared to healthy (CON) $p=0.010$, bars describe variation, and boxes median values. The abundance of CSQ illustrated by gel from western blot analysis of myalgic patients (MYA) and healthy (CON).

activity. Though, a more comprehensive analysis needs to be performed with specific antibodies analysis of the different CSQ isoforms and protein abundance in individual muscle fibers. In addition the rate at which SERCA moves Ca^{2+} across the SR membrane can be controlled by the regulatory proteins phospholamban (PLB) [57] and sarcolipin [58], reducing the rate of Ca^{2+} movement. Future studies are needed to elucidate the relationship between the different Ca^{2+} regulatory proteins and their inhibitors in myalgic muscle to understand the mechanisms of muscle pain.

In conclusion, our results show an increased abundance of fast regulatory MLC, a higher abundance of SERCA-1 proteins and a lower abundance of CSQ in myalgic muscle compared to healthy. On the basis of previous knowledge, the phosphorylations of the proteins are analyzed showing no difference in phosphorylation between individual MHC protein 2D-gel spots. Instead the MLC proteins are

characterized presenting different isoforms of the MLC. The presented results indicate differences between healthy subjects and subjects with chronic trapezius myalgia with respect to contractile molecular events. The results have to be confirmed in other studies and also elucidating if the cause e.g. pain per se, different activation pattern or deconditioning/disuse. Such knowledge will be of importance for designing targeted treatment interventions for patients with chronic myalgia. Muscle stiffness and weakness, increased muscle tension and a reduced capacity to rapidly generate force are some of the muscular symptoms of chronic work related muscle pain. Increasing the understanding regarding the biochemical mechanisms of muscle myalgia will render increased knowledge concerning the concrete muscular symptoms of myalgia.

5. Concluding Remarks

The present work contributes to the understanding of the complexity of mechanisms of muscular adaptation and especially with relevance for contractile properties due to work related muscle pain. In this study we suggest that the physiological mechanisms behind the previously shown differences in force output and endurance in muscle contraction in myalgic patients may have a relation to the pathological alterations of proteins connected to the calcium regulation of the muscle. When investigating other proteins of calcium regulation there is an evident significant difference in their abundance comparing healthy and myalgic muscle. The increased abundance of the calcium ion channel protein SERCA-1 suggests an increased cellular exchange of calcium in the myalgic muscle. In addition there is a lower abundance of the calcium buffering protein CSQ in myalgic muscle which considering previous evidence are a protein prone to cause the intrinsic contractile properties of chronic work related myalgia. Further investigations regarding calcium content in the muscle needs to be done before any conclusion regarding the calcium activity and its relation to the contractile properties of myalgic muscle can be made.

6. Supplementary material

Supplement information: Protein sequences and phosphorylated sites of detected proteins presented in table 1.

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